EXHIBIT D

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Effect of recombinant hybrid human interferon on replication and morphogenesis of HSV-1 in monkey cells

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Summary

Human recombinant alpha interferon A/D significantly reduced the replication and cell fusion induced by herpes simplex virus type 1 in monkey cells. Thin-section electron microscopy of interferon-treated monkey cells showed distinct assembly of nucleocapsids within the nucleus. Analysis of virus-specific proteins by the immunoblot technique confirmed that A/D interferon had no significant effect on the expression of major nucleocapsid proteins, although the expression of glycoproteins B and D was reduced in interferon-treated cells. The possibility of an interferon-induced block at a late stage in virus morphogenesis is discussed.

Human recombinant alpha interferon; Cell fusion; Herpes simplex virus type 1; Glycoproteins B and D; Virus morphogenesis

Herpes simplex virus, types 1 and 2 (HSV-1 and HSV-2 respectively) infections are among the most prevalent encountered by humans and have become prominent targets for potential antiviral agents. Human interferon (IFN) genes, cloned and expressed in bacteria and mammalian cells (Nagata et al., 1980; Streuli et al., 1980; Goeddel et al., 1981; McCormick et al., 1984), have recently received attention because of interferon's potential for treatment of viral infections. These IFNs have been labeled according to three families, alpha, beta and gamma with many

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subspecies varying in amino acid sequences. Furthermore, recombinant DNA experiments have generated hybrid alpha-IFN molecules which show significantly altered biologic properties. A recombinant hybrid human alpha-IFN termed A/D has recently been shown to block lethal infections caused by HSV-2 and encephalomyocarditis virus in mice (Weck et al., 1982; Fish et al., 1983) although the mechanism of action of this IFN was not identified. In addition, three human alpha interferon preparations currently being used in clinical trials, namely rIFN-αA, rIFN-α_2, and lymphoblastoid interferons, had appreciable activity against encephalomyocarditis and vesicular stomatitis viruses in guinea pig transformed and guinea pig embryo cells (Overall et al., 1984). The species specificity of IFN molecules probably is determined by cellular receptors and by the structure of the IFN molecules (Gordon and Minks, 1981). We have previously shown that cloned human alpha-2 and beta-IFNs block HSV replication in human cells at a late stage in viral morphogenesis (Chatterjee et al., 1985). In this communication, we report that recombinant hybrid human alpha-IFN, A/D, significantly inhibited the replication and multinucleate cell (syncytium) formation induced by HSV-1 in monkey cells. Furthermore, we demonstrate that the expression of specific glycoproteins was also greatly affected in these IFN-treated monkey cells, a fact which has not been previously reported. The possibilities of future studies in laboratory animals including primates as model systems is discussed.

In order to determine the effect of IFN A/D on replication of HSV-1 in heterologous system, BS-C-1 (African green monkey kidney) cells were pretreated with 0, 100 and 200 units/ml of this IFN for 18 h and then infected with the MP strain of HSV-1 at an MOI of 0.1. Supernatant fluids were collected 24 h post-infection and the quantity of virus particles released was determined by plaque assay. Table 1 summarizes the result of this experiment. Both concentrations of A/D IFN significantly (> 95%) inhibited the replication of HSV-1 in heterologous cells. In order to determine whether the observed inhibition in virus replication was due to a block in the expression of specific viral polypeptides which might affect virus assembly and transport, the following experiment was performed. African green monkey kidney cells were pretreated with 0 and 200 units/ml of A/D IFN for 18 h and then

TABLE 1

Effect of A/D IFN on the replication of HSV-1 in monkey cells [a]

IFN (units/ml)	PFU/ml	Percent control
0	3.3×10^5	100
100	1.6×10^4	4.8
200	8×10^3	2.4

[a] BS-C-1 cells, grown as described before (Chatterjee et al., 1985), were pretreated with 0, 100 and 200 units/ml of A/D (Bgl) IFN (specific activity, 1×10^8 units/mg protein; provided by P.W. Trown, Hoffmann-LaRoche Inc., Nutley, New Jersey) for 18 h and then infected with MP strain of HSV-1 as described in the text. Supernatant fluids were collected 24 h post-infection and tested for their ability to form plaques in BS-C-1 cells. Cells were then stained with May-Grunwald-Giemsa as described before (Chatterjee et al., 1985) and the plaque-forming unit (PFU) per ml calculated.

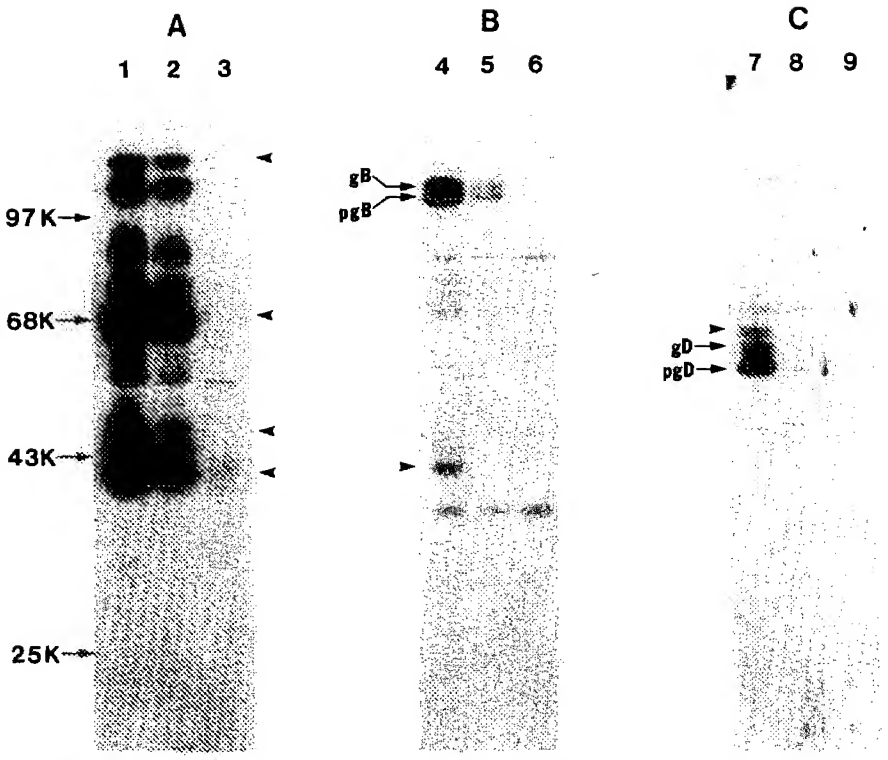
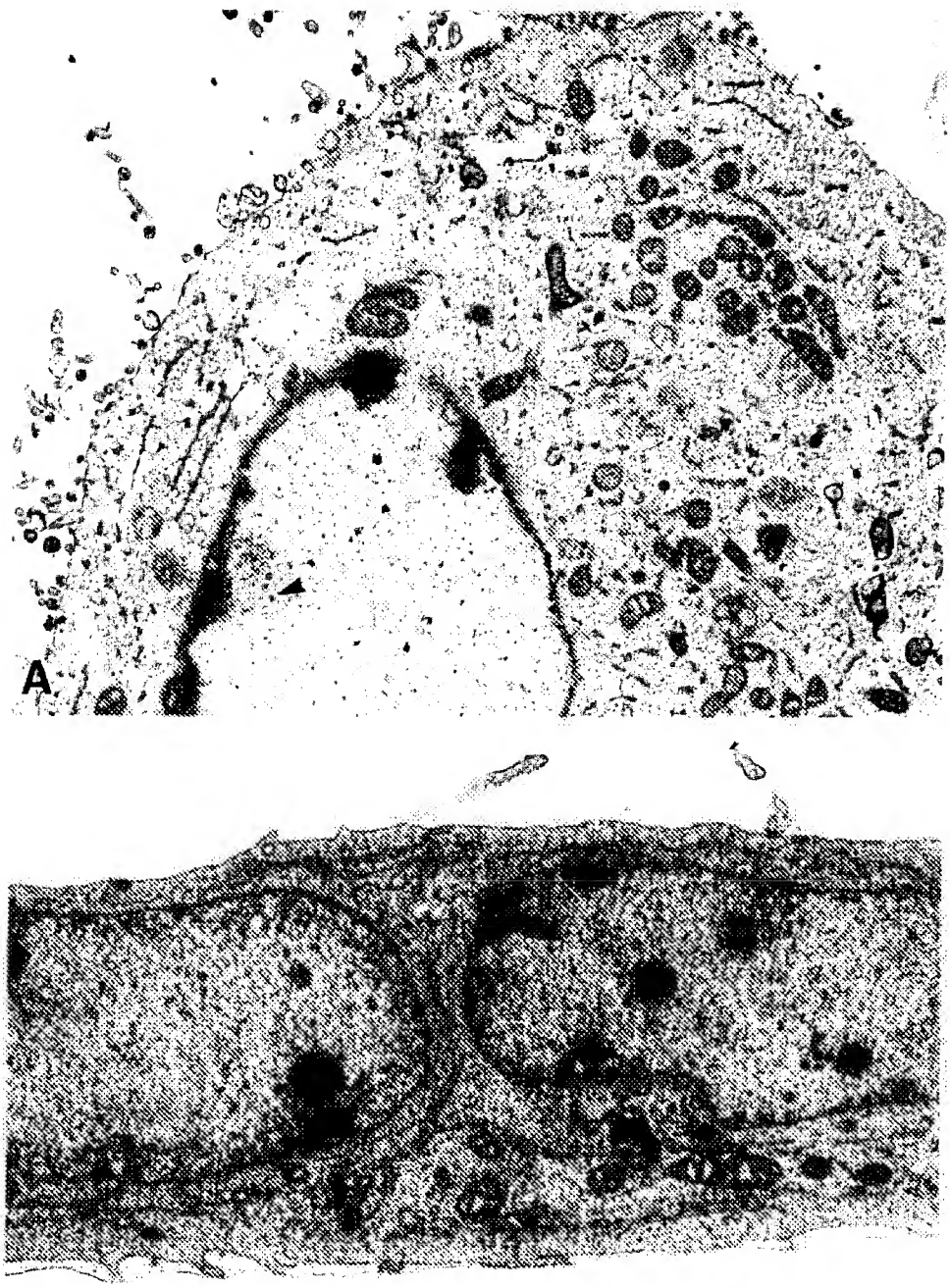


Fig. 1. Effect of A/D IFN on the expression of HSV-1 proteins in IFN-treated and untreated monkey cells. (A) BS-C-1 cells were pretreated with 0 and 200 units/ml of IFN for 18 h and then processed for immunoblotting with rabbit anti-HSV-1 antiserum after infection with MP strain as described in the text. Lane 1, no IFN; lane 2, A/D IFN; lane 3, uninfected control. The major capsid proteins are indicated by arrowheads. (B) Experimental procedure was same as above except the blot was reacted with rabbit anti-gB antiserum. Lane 4, no IFN; lane 5, A/D IFN; lane 6, uninfected control. The pgB denotes the precursor form of the glycoprotein B. Note the faster migrating extra band (arrowhead) in the untreated lane. (C) In this experiment the blot was reacted with rabbit anti-gD antiserum. Lane 7, no IFN; lane 8, A/D IFN; lane 9, uninfected control. The pgD denotes the precursor form of the glycoprotein D. Note the gD-related band (arrowhead) in the untreated lane.

infected with the MP strain as before. Cell lysates collected 18 h post-infection were processed for immunoblotting as described previously (Chatterjee et al., 1985). The blot resulting from this experiment was incubated with rabbit anti-HSV-1 antiserum (provided by B. Norrild, University of Copenhagen, Copenhagen, Denmark; Vestergaard et al., 1977) and finally reacted with [^{125}I]protein A. The result of this experiment (Fig. 1A) demonstrated that this IFN had no significant effect on the expression of major HSV-1 nucleocapsid proteins. In support of the above observation, electron microscopy of virus-infected cells showed formation of distinct nucleocapsids within the nucleus of IFN-treated cells (Fig. 2B). However, in contrast to the untreated cells (Fig. 2A), a very few extracellular mature particles were seen in the IFN-treated cells (Fig. 2B). It should be noted that some of the


A
B

nucleocapsids in the IFN-treated cells lack dense cores. Whether these structures represent an altered or modified form of DNA or entirely lack DNA, remains to be defined. In a preliminary experiment, designed to assay extracellular viral proteins by Western blots, a significant reduction in the release of extracellular particles was noticed (data not shown).

In order to explain the defect in the release of nucleocapsids from the nucleus of the IFN-treated cells, the expression of HSV-1 glycoproteins in these cells was determined by the following immunoblotting experiment. The same IFN-treated and untreated cell lysates used before were analysed for the expression of glycoproteins B and D by using rabbit antiserum against these proteins (provided by B. Norrild). The results (Fig. 1B and C) indicated that, unlike the nucleocapsid proteins, the expression of glycoproteins B and D was reduced by this IFN. However, the reduction of glycoprotein B was not as great as observed with glycoprotein D. Furthermore, in contrast to our earlier observation in human cells (Chatterjee et al., 1985), the glycoprotein profiles were slightly different in monkey cells. The glycoprotein pattern obtained after incubating with antisera against gB and gD displayed additional bands (Fig. 1B and C), which were probably intermediate or degradation products during the post-translational processing pathway as suggested by some investigators (Pereira et al., 1981, 1982; Zezulak and Spear, 1984).

Thus the block in replication appears to be at a late stage in viral morphogenesis which was further supported by the fact that this IFN significantly reduced syncytium formation in heterologous cells. In brief, BS-C-1 cells were pretreated with 0 and 100 units/ml of A/D IFN for 18 h and then infected with the MP strain as before. The infected cells stained for infection and observed for multinucleate cell formation under light microscope. The result of this experiment (Fig. 3) showed that 100 units/ml of A/D IFN almost completely blocked the syncytium formation by HSV-1 in monkey cells (Fig. 3B).

Although earlier studies showed that IFNs were species specific, subsequent development of recombinant hybrid IFN molecules clearly demonstrated a significant level of activity of these molecules in non-human cells (Weck et al., 1981; Rehberg et al., 1982). In support of these observations, we report here that cloned hybrid human IFN A/D can significantly block the replication of HSV-1 in monkey cells. This observed inhibition in replication was not due to any significant reduction in the expression of major nucleocapsid proteins as determined by immunoblotting experiment. Consistent with these observations, electron microscopy also demonstrated the presence of nucleocapsids within the nucleus of IFN-treated cells. A similar situation has also been reported in case of murine

Fig. 2. Electron microscopic observations of A/D IFN pretreated and untreated monkey cells after infection with HSV-1. Electron microscopy was carried out essentially as previously described (Chatterjee et al., 1982a). (A) Thin section of BS-C-1 cells infected with MP strain as before without IFN treatment showing extracellular and intranuclear particles (arrowheads). Magnification × 10,400. (B) Thin section of BS-C-1 cells pretreated with 200 units/ml of IFN and then infected with the same strain of HSV-1. Distinct nucleocapsids can be observed inside the nucleus (arrowheads). Magnification × 17,850.

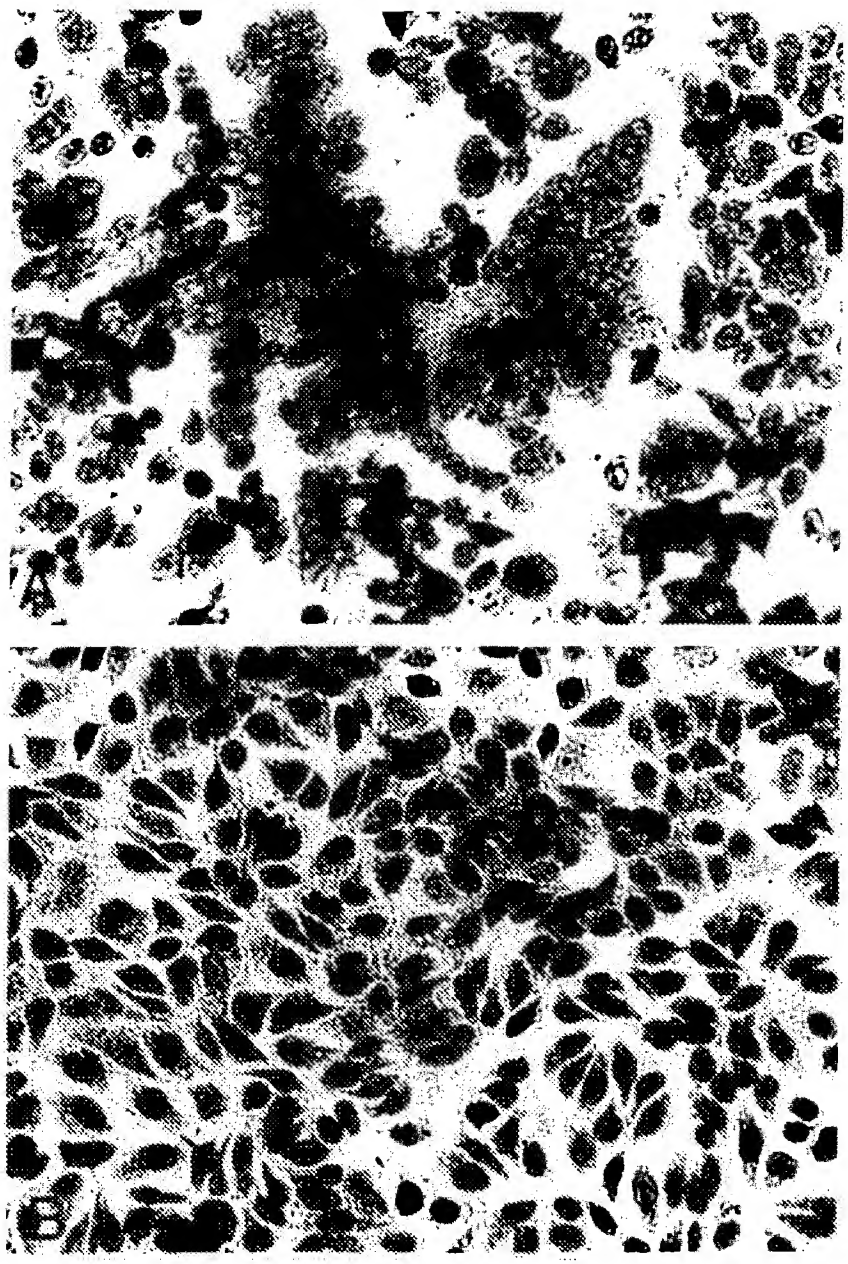


Fig. 3. Effect of A/D IFN on the multinucleate cell formation by HSV-1 in monkey cells. (A) Monolayers of BS-C-1 cells were infected with MP strain as described in the text and then stained with May-Grunwald-Giemsa 18 h post-infection as described before (Chatterjee et al., 1984). (B) In this experiment BS-C-1 cells were pretreated with 100 units/ml of A/D IFN for 18 h and then infected with MP strain and subsequently stained as above.

mammary tumor virus after IFN treatment (Sen and Sarkar, 1980). Thus, the presence of a few extracellular viral particles in IFN-treated cells, coupled with the observation of very little extracellular viral proteins suggest that A/D IFN blocked HSV-1 replication at a late stage in virus morphogenesis, i.e. in the assembly and release of nucleocapsids from the nucleus of the IFN-treated cells. Such a defect in assembly and release could be attributed to the reduced expression of glycoproteins B and D observed in the IFN-treated cells as it is possible that HSV-glycoproteins, like some other viral glycoproteins, are important for assembly and budding of nucleocapsids (Simons and Garoff, 1980; Johnson and Smiley, 1985). It should be noted that the synthesis of vesicular stomatitis virus G protein was inhibited by about 80% in transfected COS cells treated with interferon (Sahni and Samuel, 1986). At the present time, however, the possibility of an effect on other HSV-1 gene products (not quantifiable in our experiments) required for nucleocapsid assembly and budding cannot be ruled out. Alternatively, changes in the membrane fluidity which occur in IFN-treated cells (Pfeffer et al., 1981; Chatterjee et al., 1982b), could also play a role in the defect in morphogenesis. The inhibition in the expression of glycoproteins B and D might explain the significant reduction observed in multinucleate cell formation after IFN treatment. It has been previously reported that both of these glycoproteins are involved in HSV-induced cell fusion (Manservigi et al., 1977; Noble et al., 1983; Koutsoudakis et al., 1985; Ali et al., 1987). The observation that A/D IFN can significantly inhibit cell fusion in a heterologous system suggests that in vivo therapy with this IFN would prevent HSV spread from cell to cell. This information is important because multinucleate cell formation has been considered one mode by which virus can be transmitted from cell to cell (Lodmell and Watkins, 1974). Moreover, the demonstration that hybrid recombinant human IFNs can prevent replication and cell fusion in heterologous system strongly suggest for an application of these hybrid IFNs in non-human primates as a model system.

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